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Future approaches to therapeutic hypothermia: a symposium report

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Here we discuss the potential of 2 classes of novel pharmaceutical agents for decreasing core body temperature (T_b) in conscious animals. These topics were presented in a session titled *Therapeutic Hypothermia*, at the 15th International Conference on Monitoring Molecules in Neuroscience (Los Angeles, CA, August 3–7, 2014). First held in 1982 at Nottingham, UK, this biennial conference is organized by the International Society of the same name whose mission is to provide a platform to facilitate the development and refinement of methods for time-resolved detection of chemicals in the living brain. The conference was founded by pioneers in the methods of *in vivo* microdialysis, voltammetry and biosensors. Since then the meeting has evolved to include a substantial emphasis on state-of-the-art applications of neurochemical techniques. These cover a wide array of studies from single cells to whole animals such as humans. The current session demonstrates the use of wireless technologies for monitoring several physiological variables in freely moving rodents, and reviews advances in analytical techniques for the detection of adenosine which is an important neuromodulator in thermoregulation. Such techniques can be combined to investigate possible new avenues to induce, physiological, therapeutic hypothermia (TH).

Keywords: A1AR, adenosine, capillary electrophoresis, hibernation, nNOS/NK1, targeted temperature management, therapeutic hypothermia, TRPM8, torpor

Abbreviations: AGS, arctic ground squirrel; CE, capillary electrophoresis; T_b, core body temperature; EEG, electroencephalogram; FSCV, Fast scan cyclic voltammetry; HPLC, high performance liquid chromatography; ICV, intracerebroventricular; nNOS, neuronal nitric oxide synthase; NTS, *nucleus tractus solitarii*; TH, therapeutic hypothermia; TRP, transient receptor potential [channel(s)]; TRPM8, TRP melastatin-8.

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Dr. Temple Fay may have been the first pioneer in TH. In the 1930s he began experimenting with the effects of cooling inoperable tumors as well as patients with metastatic cancer or tumor growth. He found TH to be an effective treatment at relieving pain.[1] Currently, cooling is used in surgical procedures which require a cessation of blood flow during anesthesia such as in cardiopulmonary bypass surgery. The protective effects of cooling for cardiac arrest and stroke was further championed by early work in rats.[2] Mild TH is now the standard of care for some types of brain ischemia where lowering T_b to 32–34°C minimizes brain injury and improves survival and neurological outcome after cardiac arrest.[3,4] It also reduces the risk of death and disability in infants with moderate or severe hypoxic–ischemic encephalopathy.[5,6] Clinically, TH after cardiac arrest involves decreasing T_b to 32–34°C within 2h of restoration of spontaneous circulation, and maintaining reduced T_b for 12–24h. In babies with hypoxic–ischemic encephalopathy, hypothermia is maintained for 3 d.[7] Despite widespread use, the optimal target temperature for each condition remains unknown. A recent clinical study comparing cooling T_b to 33°C vs maintaining T_b at or below 36°C questions the benefit of cooling verses prevention of hyperthermia,[8] emphasizing that hyperthermia should be avoided even if colder temperatures are contraindicated. Decreasing T_b can be challenging in endotherms such as rats and humans because these species generate heat to maintain T_b near 37°C. Better understanding of the neurophysiological mechanisms that govern thermoregulation will translate to more refined and efficacious means to turn down or override the thermostat and thus manage a target T_b that is below the typical, endogenous set point of 37°C.

One novel approach for targeted temperature management, discussed by Dr. Romanovsky, is to modulate T_b and thermoeffector responses using drugs that block temperature signals driving these responses. Pharmacological blockade of thermal signals, termed by Almeida *et al.*[9] as "thermopharmacology," is based on the discoveries of several temperature-sensitive transient receptor potential (TRP) channels.[10] Some of these channels, such as melastatin-8 (TRPM8), are profoundly expressed in sensory neurons where they function as cutaneous thermosensors.[11] Pharmacological TRP antagonists block normal temperature sensing by these channels, thus making the thermoregulatory system "deaf" to environmental temperature signals. A priori, making the system insensitive to cold stimuli would be the most adequate way to prevent the undesired thermoeffector responses (nonshivering thermogenesis and shivering) in TH, but the feasibility of this approach had to be demonstrated.

Almeida et al.,[9] used M8-B, a reasonably selective and potent TRPM8

antagonist. In a mildly subthermoneutral environment (26°C vs the thermoneutral environment of 27–28°C), M8-B decreased T_b in *Trpm8*$^{+/+}$ mice and rats, but not in *Trpm8*$^{-/-}$ mice, thus suggesting an on-target hypothermic action. The authors showed that this hypothermic action was due to an attenuation of the cold-defense effector responses. M8-B also abrogated cold-induced expression of the c-Fos protein (a marker of neuronal activation) in the lateral parabrachial nucleus. The authors interpreted these results as indicating that M8-B acts within the cutaneous cold-sensing neural pathway to thermoeffectors. They propose this site to be upstream of the lateral parabrachial nucleus, most likely on primary sensory neurons. At tail skin temperatures <23°C, the magnitude of the M8-B-induced decrease in T_b was inversely related to the skin temperature in these animals, thus indicating that M8-B blocks thermal (cold) activation of TRPM8. The TRPM8 antagonist-induced hypothermia is the first example of a change in T_b of an animal occurring due to demonstrated pharmacological blockade of temperature signals at the thermoreceptor level. Other TRPM8 antagonists have also been shown to cause mild hypothermia in a cool environment.[12,13] Hence, Dr. Romanovsky proposed that thermopharmacology can be used for inducing hypothermia. Some features and limitations of TRP-based thermopharmacology are discussed elsewhere.[14]

Another method to induce hypothermia is to mimic processes involved with suppressing thermogenesis in hibernating mammals. Hibernation is a coordinated process of energy conservation utilized by a variety of mammalian species during resource limitation. One technique to enable targeted temperature management, introduced by Dr. Drew, focuses on the roles of A_1 adenosine receptors (A_1AR) as mediators of energy conservation and subsequent cooling in hibernation and torpor. Mammals that undergo torpor can be divided into 2 main groups. Obligate hibernators, such as the arctic ground squirrel (AGS), hibernate according to a seasonal rhythm. In comparison, facultative hibernators, including the hamster, hibernate in response to shortening photoperiod. Other species, like mice (such as C57BL/6J) are not true hibernators but display daily torpor when fasted. All 3 types of torpor are characterized by a decrease in the rate of oxygen consumption reflected by a decrease in heart rate and respiratory rate that precedes a subsequent decline in T_b. A_1AR activation within the CNS drives onset of hibernation or fasting-induced torpor, probably by inhibiting thermogenesis in hamsters,[15] ground squirrels,[16] and mice.[17]

Altered thermoregulation is an essential aspect of hibernation. A decrease in hypothalamic temperature is sufficient to induce thermogenesis. Early work demonstrated that the hypothalamic temperature necessary to induce thermogenesis decreased as animals entered hibernation.[18] In the AGS a seasonal change in sensitivity to A_1AR agonist underlies altered thermoregulation and the onset of spontaneous torpor. A seasonal change in sensitivity was noted initially when the A_1AR agonist (N^6-cyclohexyladenosine; CHA) induced hibernation when administered in winter but not in summer.[16] Next it was found that despite housing AGS in conditions used to minimize hibernating behavior (e.g., at an ambient temperature of 20°C and a daily light cycle of 12 h lights on and 12 h lights off), these mammals still display spontaneous torpor and seasonal fluctuation in T_b. AGS T_b was lowest during the winter season, and spontaneous bouts of torpor were seen only during these periods. Furthermore, a low dose of CHA (0.1mg/kg) that was insufficient to induce torpor, produced a decrease in T_b that was larger when administered during a period of low T_b than when administered during a period of high T_b.[19] Similarly, study of AGS in the wild revealed that altered thermoregulation predicts onset of hibernation. In a population of AGS, living free in the wild, core T_b begins to decline 45 d before onset of the first torpor bout.[20] In addition to thermoregulation,[21] adenosine contributes to homeostatic sleep drive. Thus, evidence that adenosine drives the onset of hibernation supports the hypothesis that hibernation is an extension of sleep.

Adenosine has long been implicated as the neurochemical marker for sleep homeostasis although adenosine's role in sleep is currently understood to be independent from its role in thermoregulation. Dr. Williams reviewed evidence for cortical nNOS/NK1 neurons as the cellular substrate for the sleep homeostat, with adenosine being the link for translating sleep pressure into EEG sleep-state changes by affecting cortical nNOS/NK1 neuronal excitability.

Dr. Williams explained that Radulovacki first proposed that adenosine drives the sleep "homeostat,"[22] as adenosine mimetics increase the amount of time in sleep and slow-wave activity (SWA)[23,24] or NREM delta energy.[25] SWA and NREM are both thought to be physiological indicators of sleep homeostasis. Moreover, extracellular adenosine, sampled with microdialysis, increases in the basal forebrain and cortex following extended periods of wakefulness.[26] Numerous excellent studies have attempted to identify the basal forebrain cellular substrates that interact with adenosine to mediate sleep, but the cortical substrate has proven inconclusive. To date, almost all cortical neurons are thought to be inhibited by adenosine.[27] It has been proposed that the recently identified cortical nNOS/NK1 neurons [28] may be the cortical cellular substrate mediating adenosine effects on sleep.[29] These nNOS/NK1 expressing cells are the only type of cortical cell to express c-Fos during sleep.[28,30] In addition, the proportion of nNOS neurons activated correlates to NREM delta energy.[31,32] Therefore, these cells are ideally situated to reflect cortical energy expenditure, or adenosine generation, and sleep homeostasis. However, how these neurons respond to extracellular adenosine and reset the homeostat has yet to be determined. Additionally, cortical nNOS cells may regulate cerebral blood flow,[33,34] which is an important determinant of local brain temperature. In rodents, brain temperature increases during sleep deprivation and decreases during recovery sleep during which there is a subsequent increase in NREM activity.[35,36,37] Therefore, cortical nNOS neurons may also play a role in restoring brain temperature following extended wakefulness. However, there is also evidence that nNOS activation during ischemic damage, such as stroke, has detrimental effects on neuronal recovery. Hence, understanding the role

for cortical nNOS cells in coupling sleep, adenosine, and brain thermoregulation could provide a novel therapeutic target for controlling T_b.

Dr. Tupone, whose conference participation was sponsored by Data Sciences International, reviewed evidence that intracerebroventricular (ICV) administration of the A_1AR agonist, CHA, induces a hypothermic, torpor-like response in male Wistar rats.[38] Inhibition of thermogenesis from both shivering and brown adipose tissue (BAT) was shown to contribute significantly to the hypothermia caused by CHA. Further, the thermogenolytic action of CHA was attributable to its actions specifically within the *nucleus tractus solitarii* (NTS). These results are consistent with the hypothesis that an NTS-mediated inhibition of thermogenesis plays a hypothermic role in CHA-induced torpor and in spontaneous (i.e., endogenous adenosine-induced) hibernation. In his study,[38] ICV administration of CHA in freely-behaving rats exposed to a cool (15°C) ambient temperature, produced a torpor-like state similar to that in hibernating species. This torpor-like state was characterized not only by a fall in T_b without activation of BAT or shivering thermogenesis, but also by a marked reduction in EEG amplitude and a large bradycardia, also consistent with hibernation-like behavior. Skipped heartbeats and transient bradycardias were vagally-mediated as they were eliminated by systemic muscarinic receptor blockade. Upon subsequent rewarming, rats in this torpor-like state regained normothermic homeostasis. Revealing the neurophysiological basis for the thermogenolytic responses to CHA, ICV or local NTS administration of CHA inhibited skin cooling-evoked increases in BAT sympathetic nerve activity and in shivering EMG activity. These results are consistent with the demonstration of a greater number of c-Fos-expressing neurons within NTS after centrally administered CHA. Together, these findings demonstrate that a deeply hypothermic, torpor-like state can be pharmacologically induced in a non-hibernating mammal and that recovery of normothermic homeostasis ensues upon rewarming. These results also support the potential utility of a centrally-acting A_1AR agonist for the induction of a clinically useful therapeutic hypothermia.[39,40]

It is apparent that adenosine plays a prominent role in thermoregulation, hibernation and sleep. Therefore monitoring changes in adenosine and adenosine metabolites, is crucial to the development of TH. Ms. Stephen discussed current techniques and pitfalls for monitoring adenosine in biological fluids. To date, the most commonly used technique for analyzing adenosine off-line is high performance liquid chromatography (HPLC) coupled to UV-Vis detection.[26,41] Recent studies have strived to increase the sensitivity of HPLC by developing nanoscale separation columns and coupling the method to mass spectrometry and electrochemical detectors.[42,43] While HPLC sensitivity has improved and HPLC can be used to measure adenylate metabolites as well as adenosine, one drawback of HPLC is that it requires relatively large injection volumes that can limit use of this method if good temporal resolution is a priority.

Another technique used for monitoring adenosine is capillary electrophoresis (CE) with laser induced fluorescence detection.[44] CE overcomes some of the limitations of HPLC because it requires only nanoliter sample volumes, and has high separation efficiency for adenosine, thus, CE allows for greater sensitivity and resolution than HPLC. However, a disadvantage of the CE method is that the derivation reactions, necessary to enhance sensitivity and detection of physiological concentrations of adenosine, require processing at high temperatures. Recent research has also focused on electrochemical [fast scan cyclic voltammetry (FSCV)] and enzyme-based biosensor technology to monitor adenosine in real-time.[45,46] These techniques are advantageous since adenosine can be directly monitored on a sub-second time scale. However, they are not robust methods to monitor adenosine when brain temperature varies since the mechanism of signal generation is affected by temperature, potentially in a nonlinear manner. Moreover, both CE and HPLC offer an opportunity to measure a range of adenylates and related compounds and, relative to biosensors and FSCV, are less vulnerable to signal interference from other biological compounds with similar oxidation potentials such as hydrogen peroxide and ATP.[46] Therefore, although there are limitations with each method, together they provide the capability to increase our understanding of adenosine's role in thermoregulation and possibly aid in the development of new methods for the induction of TH.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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